

## DESCRIPTION

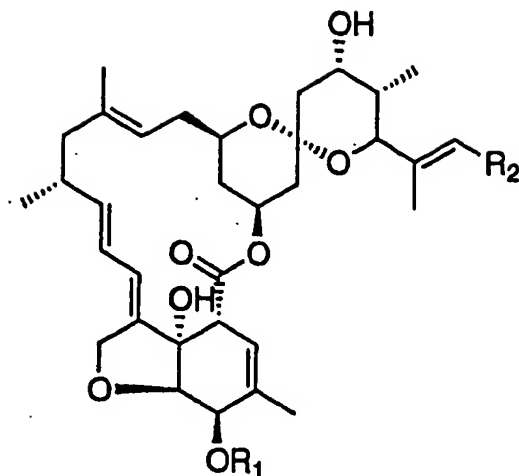
A strain having C-13 substituted nemadectin producing activity belonging to genus Streptomyces and a method for manufacturing  
5 c-13 substituted nemadectin using the same

## Technical Field

The present invention relates to a strain having C-13 substituted nemadectin producing activity belonging to genus  
10 Streptomyces and a method for manufacturing C-13 substituted nemadectin using the same. More particularly, the present invention pertains to the method for manufacturing C-13 hydroxynemadectin and C-13 glycosynemadectin using the microorganism belonging to genus Streptomyces having C-13  
15 substituted nemadectin producing activity, and the microorganism strain belonging to Streptomyces cyaneogriseus subspecies noncyanogenus.

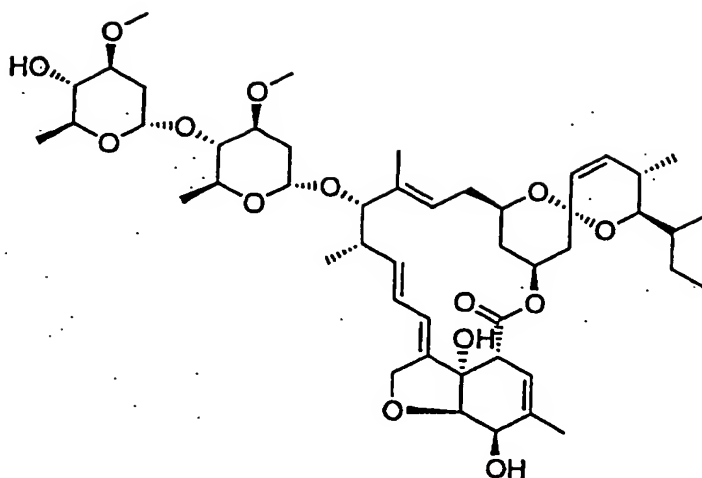
## Background Art

20 A series of compounds having benzofuran ring structure has excellent antiparasitic activity and antiinsect activity. Among them, avermectin and milbemycin are now practically used. Four components,  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ , of nemadectin, which has benzofuran ring structure produced by Streptomyces cyaneogriseus  
25 subspecies noncyanogenus, are known, and the C-13 position thereof has no substituent and is saturated as shown in the following structure.



	Nemadectin $\alpha$	$R_1 = H$	$R_2 = CH(CH_3)_2$
	Nemadectin $\beta$	$R_1 = H$	$R_2 = CH_3$
5	Nemadectin $\gamma$	$R_1 = CH_3$	$R_2 = CH_3$
	Nemadectin $\delta$	$R_1 = CH_3$	$R_2 = CH(CH_3)_2$

A reason why the C-13 position of nemadectin is saturated  
 is that a module 7 of nemadectin polyketide synthetase  
 (nemadectin PKS), which is involved in the formation of  
 10 nemadectin aglycon moiety, is constructed by the structure of  
 KS-AT-DH-ER-KR-ACP. It is difficult to construct  
 stereoselective modification in the saturated C-13 position by  
 chemical synthesis. Although increasing antiinsect activity and  
 15 antiparasitic activity can be expected by an addition of sugar  
 moiety as like in avermectin of the following structure, a  
 production of derivatives by chemical synthesis has not been  
 made.



As described in the above, although stereoselective introduction of hydroxyl group and glycosidation of the hydroxyl group of nemadectin at C-13 position by chemical synthesis might be difficult to perform, as a result of extensive studies, we have succeeded in preparing C-13 glycosidated nemadectin producing microorganism by means of the molecular genetic technology and obtaining efficiently nemadectin with stereoselective glycosidation.

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The present invention was completed based on such the knowledge. An object of the present invention is to provide a microorganism belonging to genus *Streptomyces* having C-13 glycosylnemadectin producing activity by the molecular genetic technology. Another object of the present invention is to provide a microorganism strain belonging to genus *Streptomyces* having C-13 substituted nemadectin producing activity, which can be used for obtaining effectively nemadectin with stereoselective glycosidation and expected to improve the biological activity thereof.

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Further object of the present invention is to provide a method for manufacturing C-13 substituted nemadectin comprising introducing DNA of a microorganism, which produces nemadectin analog, into the nemadectin producing microorganism belonging to genus *Streptomyces* and accumulating C-13 hydroxynemadectin and C-13 glycosynemadectin and collecting the same.

#### Disclosure of the Invention

We have prepared the C-13 hydroxynemadectin producing microorganism strain by modifying gene groups of nemadectin aglycon biosynthesis in order to obtain C-13 hydroxynemadectin, which can be modified for adding the sugar moiety by chemical synthesis, and generating a hybrid polyketide synthetase (hybrid PKS) with nemadectin PKS and avermectin polyketide synthetase (avermectin PKS). Further, we have improved a productivity of C-13 hydroxynemadectin as a result of stimulating transcription of avermectin PKS gene by introducing *aveR* gene which was involved in the transcriptional control of avermectin PKS.

The present strain *Streptomyces cyaneogriseus* subsp. *noncyanogenus*  $\Delta$ nemA4::vph attB<sub>TG1</sub>::aveA4-aveA3-aveE attB<sub>φ<sub>c31</sub></sub>::aveR was deposited in the International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, 305-8566 Japan based on Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure as accession number FERM BP-8395 on June 6th, 2003.

Further, we have prepared the microorganism strain, to which aveBI-BVIII gene group involving in glycosidation of avermectin and oleandrose biosynthesis was introduced, and prepared the C-13 glycosylnemadectin producing microorganism strain.

The present strain *Streptomyces cyaneogriseus* subsp. noncyanogenus  $\Delta$ nemA4::vph attB<sub>TG1</sub>::aveA4-aveA3-aveE attB $\phi$ <sub>C31</sub>::aveR attB<sub>R4</sub>::aveB1-BVIII was deposited in the International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, 305-8566 Japan based on Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure as accession number FERM BP-8394 on June 6th, 2003.

The present invention relates to a method for manufacturing C-13 glycosylnemadectin comprising culturing a microorganism strain belonging to *Streptomyces cyaneogriseus* subsp. noncyanogenus, producing and accumulating C-13 glycosylnemadectin and isolating C-13 glycosylnemadectin from the cultured mass. Further, the present invention relates to the microorganism strain belonging to *Streptomyces cyaneogriseus* subsp. noncyanogenus and having ability to produce C-13 hydroxylnemadectin and C-13 glycosylnemadectin.

As described hereinabove, a report, wherein C-13 hydroxylnemadectin and C-13 glycosylnemadectin were produced

and accumulated by introducing DNA of the microorganism, which produced nemadectin analogous compounds, into the nemadectin producing microorganism belonging to genus *Streptomyces*, has not been known.

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Consequently, the present invention provides the microorganism strain belonging to *Streptomyces cyaneogriseus* subsp. *noncyanogenus* and having ability to produce C-13 glycosidated nemadectin.

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Further, the present invention provides the microorganism strain belonging to *Streptomyces cyaneogriseus* subsp. *noncyanogenus* and having ability to produce C-13 hydroxylnemadectin.

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Further, the present invention provides a method for manufacturing C-13 hydroxylnemadectin comprising culturing a microorganism strain belonging to *Streptomyces cyaneogriseus* subsp. *noncyanogenus* and having ability to produce C-13 hydroxylnemadectin, producing and accumulating C-13 hydroxylnemadectin in the cultured medium and isolating C-13 hydroxylnemadectin from the cultured mass.

Further, the present invention provides a method for manufacturing C-13 glycosidated nemadectin comprising culturing a microorganism strain belonging to *Streptomyces cyaneogriseus* subsp. *noncyanogenus* and having ability to produce C-13 glycosidated nemadectin, producing and accumulating C-13 glycosidated nemadectin in the cultured medium and isolating

C-13 glycosidated nemadectin from the cultured mass.

Further, the present invention provides a microorganism strain belonging to *Streptomyces cyaneogriseus* subsp. noncyanogenus, maintaining gene groups of avermectin aglycon biosynthesis of *Streptomyces avermitilis* and having ability to produce C-13 hydroxylnemadectin, and a method for preparation of the microorganism.

Further, the present invention provides a microorganism belonging to *Streptomyces cyaneogriseus* subsp. noncyanogenus, maintaining gene groups of avermectin aglycon biosynthesis of *Streptomyces avermitilis* and having ability to produce C-13 glycosidated nemadectin, and a method for preparation of the microorganism.

Further, the present invention provides a nemadectin non-producing microorganism strain belonging to *Streptomyces cyaneogriseus* subspecies noncyanogenus and inserting viomycin resistant gene in the region coding nemadectin aglycon biosynthesis genes nemA3-4 operon KS10 (KS10 insertion mutant).

Further, the present invention provides a microorganism strain belonging to *Streptomyces cyaneogriseus* subspecies noncyanogenus, maintaining avermectin aglycon biosynthesis genes aveA3-4 of *Streptomyces avermitilis* in the KS10 insertion mutant, and having ability to form a hybrid PKS with NemA1-2 and AVES3-4.

Further, the present invention provides a microorganism strain belonging to *Streptomyces cyaneogriseus* subspecies noncyanogenus and having ability to form a hybrid PKS with NemA1-2 and AVES3-4, wherein the microorganism strain maintains  
5 a regulator gene aveR of avermectin biosynthesis genes of *Streptomyces avermitilis*.

Further, the present invention provides a microorganism strain belonging to *Streptomyces cyaneogriseus* subspecies  
10 noncyanogenus and having ability to form a hybrid PKS with NemA1-2 and AVES3-4, wherein the microorganism strain maintains a regulator gene aveR of avermectin biosynthesis genes and an avermectin glycosidation and an oleandrose biosynthesis genes aveBI-BVIII of *Streptomyces avermitilis*.

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#### Brief description of drawings

Fig. 1 is a restriction map of 3.0 kb fragment containing KS region of nemadectin PKS module 10. Arrow indicates a direction for transcription.

20 Fig. 2 is a restriction map of an insertion fragment of SalI region vph in nemadectin KS 10 region. Arrow indicates a direction for transcription.

Fig. 3 is  $^1\text{H}$ -NMR spectrum of C-13 hydroxylnemadectin.

Fig. 4 is  $^{13}\text{H}$ -NMR spectrum of C-13 hydroxylnemadectin.

25 Fig. 5 is  $^1\text{H}$ -NMR spectrum of C-13 glycosylnemadectin.

Fig. 6 is  $^{13}\text{H}$ -NMR spectrum of C-13 glycosylnemadectin.

#### Best Mode for Carrying out the Invention

The present invention will be explained concretely by



following examples, but the present invention is not limited within the description of these examples.

#### Example 1

Obtaining *Streptomyces cyaneogriseus* subsp. *noncyanogenus*  
5 NRRL 15773, to which viomycin resistant gene (viomycin phosphotransferase: *vph*) was inserted into KS region of nemadectin PKS.

(1) Subcloning of DNA fragment coding nemadectin PKS, KS 10  
10 A cosmid DNA containing DNA coding a KS domain (NEM-KS10) of the module 10 in the cosmid DNA containing nemadectin aglycon synthetase gene was digested by a restriction enzyme BamHI (TAKARA BIO INC., Japan) and was electrophoresed with agarose gel. A DNA fragment 3.0 kb containing KS 10 region was isolated  
15 and purified by using a gene clean II kit (Bio101 Inc., US). In addition, a plasmid pUC 19 (TAKARA BIO INC., Japan) was digested with BamHI and was dephosphorylated by using alkaline phosphatase (Calf intestine) (TAKARA BIO INC., Japan). The 3.0 kb fragment containing NEM-KS10 and the BamHI digestion product  
20 of pUC19, each about 0.1  $\mu$ g, were ligated by reacting at 16°C for 16 hours with using Ligation High (TOYOBO CO. LTD., Japan).

The DNA ligation product 10  $\mu$ l and competent cells of *E. coli* DH5 $\alpha$  (Nippon Gene K.K., Japan) were contacted to perform  
25 transformation. LB agar medium 20 ml containing ampicillin 50  $\mu$ g/ml (Wako Pure Chemicals Inc., Japan) was used for selection of transformant strains. Aqueous solution of isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) 0.1 mol/lit. and dimethylformamide (Nacalai Tesque Inc., Japan) solution of 2%

5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-gal, Nacalai Tesque Inc., Japan), each 50  $\mu$ l, were previously smeared. Since a colony of the transformant maintaining a recombinant plasmid is defective in  $\beta$ -galactosidase activity, it can not decompose X-gal to exhibit leucoform. The leucoform colony was collected by using a loop, inoculated into LB medium 10 ml, and shake cultured at 37°C for 16 hours, then plasmids were extracted from bacterial cells and purified by alkaline method. A part of the thus obtained recombinant plasmids was digested with the restriction enzyme BamHI to confirm obtainment of plasmid pUC 19::NEM-KS10, in which DNA fragment 3.0 kb was inserted into the pUC 19.

(2) Determination of a terminal sequence of BamHI DNA fragment 3.0 kb derived from *Streptomyces cyaneogriseus* subsp. noncyanogenus NRRL 15773

At first, a template DNA for the cycle sequencing was prepared. A primer set consisting of Expand Taq DNA polymerase buffer (Roche Inc., U.S.), dATP, dGTP, dCTP, dTTP, a synthetic DNA having base sequence of 5'-GTGCTGCAAGGCGATTAAGTTGG-3' described in SEQ ID NO:1 and a synthetic DNA having base sequence of 5'-TCCGGCTCGTATGTTGTGTGGA-3' described in SEQ ID NO:2 was added to the recombinant plasmid pUC19::NEM-KS10 obtained in example 1-(1), and Expand Taq DNA polymerase (Roche Inc. U.S.) was added thereto, then a reaction consisting of a cycle at 96°C for 30 sec. and at 70°C for 3 min. was repeated for 30 cycles. After completion of the reaction, exonuclease I (Amersham Pharmacia Biotech Inc., U.S.) and alkaline phosphatase (Amersham Pharmacia Biotech Inc., U.S.) were added thereto and reacted

at 37°C for 15 min., then treated at 80°C for 10 min. for denaturing the enzymes. After denature of both enzymes, the cycle sequencing reaction was conducted DNA by adding IR labeled primer (Aloka Co. Ltd., Japan) and Thermo sequenase Fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech Inc., U.S.) were added with using the template DNA of the above. After the reaction was completed, the reaction terminator was added and mixed to prepare the sample solution.

10        The sample solution was heated at 90°C for 2 min. and ice-cooled, then the sequence electrophoresis was conducted. DNA sequencer Model 4000 Series (LI-COR Inc., U.S.) was used as an electrophoresis apparatus. Imaging analysis after the electrophoresis was performed by using Image Analysis Ver. 2.10  
15    of Base Image IR Software Ver. 2.30. Based on the thus obtained each base sequence of DNA fragments, the amino acid sequences were detected by using BLAST. As a result, a sequence having high homology with *S. avermitilis* aveA4 in one end and a sequence having high homology with reductase of *S. avermitilis* in the  
20    other end were found. From these base sequences, a transcriptional direction of the nemadectin PKS gene of BamHI fragment was confirmed (refer to Fig. 1).

(3)    Insertion of viomycin resistant gene (viomycin  
25    phosphotransferase; vph) in NEM-KS10 region

A plasmid pUC19::NEM-KS10 was digested with a restriction enzyme BamHI, and the digested mixture was treated by agarose gel electrophoresis, then DNA fragment 3.0 kb containing KS10 region was isolated and purified. A DNA fragment, about 3.0 kb,

0.1 µg obtained by digesting pBluescript SK+ (TOYOBO CO. LTD., Japan) and BamHI digestion fragment 0.1 µg containing NEM-KS10 were mixed. The mixture was ligated with a reaction at 16°C for 16 hours by using Ligation High (TOYOBO CO. LTD., Japan). The transformation was performed by contacting with the DNA ligation product 10 µl and the competent cell of E. coli DH5α to obtain the recombinant plasmid pBluescript SK+:NEM-KS10 which was the ligated plasmid with pBluescript SK+ and NEM-KS10 fragment. Further, the pBluescript SK+:NEM-KS10 was digested with the restriction enzymes HindIII (TAKARA BIO INC., Japan) and SstI (GIBCO BRL Inc., U.S.) and electrophoresed with agarose gel to obtain a DNA fragment containing NEM-KS10, about 3.0 kb. A plasmid pUC19 was digested with HindIII and SstI to obtain a DNA fragment, about 2.7 kb. Both DNA fragments, each 0.1 µg, were mixed and ligated with a reaction at 16°C for 16 hours by using Ligation High. Using the DNA ligation product 10 µl, E. coli DH5α was transformed to obtain the plasmid pUC19-Bgl::NEM-KS10, which was the plasmid ligating NEM-KS10 fragment to pUC19-Bgl (inserting BglII cleavage sequence AGATCT into the outside of the both end EcoRI and HindIII of the multicloning site of pUC19).

A vph was obtained by digesting the plasmid pUC19::vph with a restriction enzymes EcoRI (TAKARA BIO INC., Japan) and PstI (TAKARA BIO INC., Japan), electrophoresing with agarose gel and isolating and purifying the DNA fragment, 1.7 kb, containing vph. A blunt end of EcoRI/PstI DNA fragment, 1.7 kb, containing vph was obtained by using BKL kit (TAKARA BIO INC., Japan) with a reaction at 37°C for 15 min. After digesting the

pUC19-Bgl::NEM-KS10 with the restriction enzyme SalI (TAKARA BIO INC., Japan), the blunt end of SalI cleavage site was prepared by using BKL kit. The fragments with blunt end and DNA fragment, 1.7 kb, with blunt end of the above were mixed, and ligation of DNA was performed by using Ligation High. E. coli DH5 $\alpha$  was transformed by using the DNA ligation product 10  $\mu$ l to obtain the recombinant plasmid pUC19-Bgl::NEM-KS10-vph, in which vph was inserted into the KS10 region (refer to Fig. 2). A selection of the transformant was performed by using LB medium containing ampicillin 50  $\mu$ g/ml and tuberactinomycin N 150  $\mu$ g/ml.

After digesting pUC19-Bgl::NEM-KS10-vph with the restriction enzyme BglII (TAKARA BIO INC., Japan), the product was electrophoresed with using agarose gel to isolate and purified the DNA fragment, 4.7 kb, containing KS10-vph region. After a vector plasmid pGM160 for Streptomyces was digested with the restriction enzyme BamHI, the product was electrophoresed with using agarose gel to isolate and purified the DNA fragment, 6.8 kb. Further, 5' terminal of DNA was dephosphorylated by using alkaline phosphatase (calf intestine). BamHI digested product of the pGM160 and DNA fraction, 4.7 kb, containing NEM-KS10-vph region, each 0.1  $\mu$ g, were mixed to ligate DNA by using Ligation High. E. coli DH5 $\alpha$  was transformed by using the DNA ligated product 10  $\mu$ l to obtain the recombinant plasmid pGM160::NEM-KS10-vph. A selection of the transformant was performed by using LB medium containing ampicillin 50  $\mu$ g/ml and tuberactinomycin N 150  $\mu$ g/ml. Using the pGM160::NEM-KS10-vph, E. coli GM2929 hsdS::Tn10 was transformed. A selection of the transformant was performed by using LB medium containing

chloramphenicol (Wako Pure Chemicals Inc., Japan) 30 µg/ml, ampicillin 50 µg/ml and tuberactinomycin N 150 µg/ml. A non-methylated plasmid DNA pGM160::NEM-KS10-vph was prepared from the transformant of *E. coli* GM2929 hsdS::Tn10.

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(4) A preparation of protoplast from *Streptomyces cyaneogriseus* subsp. *noncyanogenus* NRRL 15773

Spore suspension 50 ml of lyophilized (at -30°C) *Streptomyces cyaneogriseus* subsp. *noncyanogenus* was inoculated into YEME medium (in 500 ml Erlenmeyer flask) containing 30% w/v sucrose, 0.5% w/v glycine and 5 mM MgCl<sub>2</sub>, and cultured at 30°C for 48 hours by rotary shaker. Bacterial cells were collected by centrifugation at 3000 rpm for 10 min. and the bacterial cells were washed with centrifugation for 10 min. A P10 medium containing egg lysozyme 1 mg/ml was added to the washed bacterial cells and suspended to form protoplasts at 30°C for 30 minutes. After well mixing with adding P10 medium 10 ml, the protoplasts suspension was filtered through a cotton plug filter to remove lysozyme indigested mycelia. The protoplast suspension passed through the cotton plug filter was centrifuged at 3000 rpm for 10 min. to precipitate the protoplasts. Supernatant was removed and the precipitate was well suspended with P10 medium 10 ml, and the suspension was centrifuged at 3000 rpm for 10 min. to precipitate the protoplasts. The P10 medium 10 ml was added again to the precipitate to suspend the protoplasts, and the protoplasts were washed by centrifugation. The thus obtained washed protoplasts were suspended in the P10 medium 5 ml. The suspension, each 0.1 ml, was dispensed into Eppendorf tube and stored at -80°C.

(5) Preparation of gene recombinant to which viomycin resistant gene (viomycin phosphotransferase; vph) is introduced in KS10 region of nemadectin PKS on the chromosome

5       The recombinant plasmid pGM160::NEM-KS10-vph, about 1 µg, obtained in example 1-(3) and protoplasts, about  $5 \times 10^8$ , of *Streptomyces cyaneogriseus* subsp. *noncyanogenus* obtained in example 1-(4) were poured in a sterilized Eppendorf tube, and immediately added and mixed with 25% polyethylene glycol MW1000  
10       solution (2.5% sucrose, 0.05%  $\text{KH}_2\text{PO}_4$ , 0.1 M  $\text{CaCl}_2$  and 50mM Tris-maleate, pH 8.0) and allowed to stand at room temperature for 1 min. After P10 medium 450 µl was added and mixed well, each 100 µl thereof was placed on R2YE agar medium and was spread over together with soft agar medium 2.5 ml. After incubating  
15       at 30°C for 20 hours, a soft agar medium 2.5 ml containing thiostrepton (Sigma-Aldrich Co., U.S.) 200 µg/ml was overlaid. The medium was cultured at 30°C for and the transformant resistant to thiostrepton was obtained.

20       The transformant resistant to thiostrepton grown on the surface of R2YE agar medium was scratched aseptically. Mycelia were cut by using homogenizer and the cleaved mycelia were spread over on YMS agar medium. The medium was cultured at 37°C for 4 days, and sporogenous mycelia were replicated on the YMS agar  
25       medium containing tuberactinomycin N as a master plate. The plate was cultured at 30°C for 2 days, and tuberactinomycin resistant colonies were selected, then each colony was spread over on YMS agar medium. The medium was cultured at 30°C for 5 days, and sporogenous mycelia were replicated on the YMS agar medium

containing thiostrepton 20µg/ml as a master plate, then the plate was cultured at 30°C for 2 days. Strains resistant to tuberactinomycin and sensitive to thiostrepton were selected and confirmed the insertion of the vph in the KS10 region of nemadectin PKS on the chromosome by means of Southern hybridization as well as confirming no production of nemadectin. The thus obtained each strain was referred to *Streptomyces cyaneogriseus* subsp. *noncyanogenus* ΔnemA4::vph.

10       The present strain *Streptomyces cyaneogriseus* subsp. *noncyanogenus* ΔnemA4::vph was deposited in the International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, 305-8566 Japan based  
15       on Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure as accession number FERM BP-8393 on June 6th, 2003.

(6) Obtaining avermectin synthetase gene aveA3 derived from  
20       *Streptomyces avermitilis*

A chromosomal DNA of *Streptomyces avermitilis* was digested with the restriction enzyme EcoRI and electrophoresed with low melting point agarose gel. A DNA fragment, 39912 bp, described in SEQ ID NO:3 containing total aveA3-4 was cut out with gel.  
25       The gel was isolated and purified by means of phenol extraction, phenol-chloroform extraction and alcohol precipitation. In addition, a chromosome inserted vector plasmid pTGlint-cos was digested with the restriction enzyme EcoRI and electrophoresed with agarose gel to isolate and purify DNA fragment, 5.2 kb.



The EcoRI digested pTGling-cos was dephosphorylated at 5' terminal of the DNA by using alkaline phosphatase (calf intestine), and about 0.5 µg thereof was mixed with the DNA fragment, 39912 bp, about 2 µg, containing the total aveA3-4, and was ligated with the reaction at 25°C for 10 min. by using Ligation kit ver. 2 (TAKARA BIO INC., Japan) solution I and solution II.

After the DNA ligate was treated with alcohol precipitation, the DNA was dissolved in TE buffer 2 µl. The solution was added to Packaging Extract of ReadyToGo Lambda Packaging Kit (Amersham-Pharmacia Biotech, Inc., U.S.), and added sterilized water 23 µl, then allowed to stand at room temperature for 2 hours. A phage diluted buffer (SM buffer) 0.5 ml and chloroform 30 µl were added thereto and mixed by gentle tumbling. The mixture was centrifuged at 13200 rpm for 30 sec. and the supernatant was transferred to a new sterilized Eppendorf tube to obtain λ phage packaging solution.

(7) Obtaining E. coli BL21 recA deficient strain maintaining avermectin aglycon synthetase gene aveA3-4

Using the λ phage packaging solution obtained in example 1-(6), a transduction was performed with the host cell E. coli BL21 recA deficient strain. The host cell E. coli BL21 recA deficient strain was shake cultured with LB medium at 37°C for overnight, and was added to LB medium added with 0.4% maltose to become 1% and cultured 37°C for 3 hours. Cells were collected by centrifugation and were washed with using 10 mM magnesium sulfate solution. Bacterial cells were further collected by

centrifugation and were suspended in adequate amount of 10 mM magnesium sulfate solution to obtain the host bacterial cell solution. The host bacterial cell solution and the  $\lambda$  phage packaging solution obtained in example 1-(6) were mixed at a  
5 rate 1 : 1 in an Eppendorf tube, then allowed to stand at room temperature for 30 minutes. Thereafter, LB medium was added and shaken at 30°C for 1.5 hour, then the cultured medium was spread on the LA medium containing kanamycin (50  $\mu$ g/ml) and cultured at 30°C for overnight. Colonies resistant to kanamycin were  
10 cultured in 96 well test plate as a library, and clones maintaining cosmid DNA hybridized with the synthetic DNA described in SEQ ID NO:4 were selected. The recombinant DNA maintaining avermectin aglycon synthetase gene aveA3-4 was purified from the bacterial cells which were cultured for  
15 overnight by using LB medium containing kanamycin (50  $\mu$ g/ml) according to the conventional alkaline method.

## Example 2

Introduction of avermectin biosynthesis genes aveA3-4 into  
20 nemadectin PKS module 10vph

The spore suspension of nemadectin PKS module 10vph insertion strain obtained in example 1-(5) was inoculated into YEME medium (500 ml Erlenmeyer flask) containing 50 ml of 30% w/v sucrose, 0.5% w/v glycine and 5mM MgCl<sub>2</sub>, and cultured at 30°C  
25 for 48 hours in the rotary shaker. Mycelia were collected by centrifugation at 3000 rpm for 10 min. After P10 medium 20 ml was added and suspended well, the suspension was centrifuged at 3000 rpm for 10 min. to wash mycelia. The P10 medium containing egg lysozyme 1 mg/ml was added to the washed mycelia to suspend

and to generate the protoplast by keeping at 30°C for 30 minutes. After well mixing with adding P10 medium 10 ml, the protoplast suspension was passed through a cotton plug filter to remove lysozyme indigested mycelia. The protoplast suspension which  
5 was passed through the cotton plug filter was centrifuged at 3000 rpm for 10 min. to precipitate the protoplast. After removing the supernatant and suspending well with P10 medium 10 ml, the protoplast was precipitated by centrifuging at 3000 rpm for 10 min. The P10 medium 10 ml was again added thereto  
10 and the protoplast was suspended and washed by centrifugation. The obtained washed protoplast was suspended in P10 medium 5 ml. The suspension, each 0.1 ml, was dispensed in the sterilized Eppendorf tube and stored at -80°C.

15       The pTGlint-cos::aveA3-4, about 1 µg, prepared in example 1-(7) was added to the protoplast hereinabove and immediately added and mixed with 25% polyethylene glycol MW1000 solution (2.5% sucrose, 0.05% KH<sub>2</sub>PO<sub>4</sub>, 0.1 M CaCl<sub>2</sub> and 50mM Tris-maleate, pH 8.0) 500 µl and allowed to stand at room temperature for 1  
20 min. After P10 medium 450 µl was added and mixed well, each 100 µl thereof was placed on R2YE agar medium and was spread over together with soft agar medium 2.5 ml. After incubating at 30°C for 20 hours, a soft agar medium 2.5 ml containing neomycin (Sigma-Aldrich Co., U.S.) 100 µg/ml was overlaid. The medium  
25 was cultured at 30°C for 5 days to obtain the transformant resistant to neomycin. The transformant resistant to neomycin grown on the surface of R2YE agar medium was spread aseptically over the YMS agar medium containing neomycin 2 µg/ml. The thus obtained each strain was referred to *Streptomyces cyaneogriseus*

subsp. noncyanogenus  $\Delta$ nemA4::vph attB<sub>TG1</sub>::aveA4-aveA3-aveE.

### Example 3

Introduction of aveR to *Streptomyces cyaneogriseus* subsp.

5 noncyanogenus  $\Delta$ nemA4::vph attB<sub>TG1</sub>::aveA4-aveA3-aveE

The vector plasmid pUCBM21::aveR ligated with the restriction enzyme AgeI DNA fragment containing the transcriptional regulatory gene aveR of avermectin biosynthesis genes described in SEQ ID NO:5 was digested with restriction  
10 enzymes XbaI and HindIII, and electrophoresed with agarose gel to isolate and purify DNA fragment, 3.27 kb, containing aveR. The XbaI-HindIII fragment, 3.27 kb, containing aveR was ligated into XbaI and HindIII recognition sites of the chromosomal integrative vector plasmid pUC19aad3"-int $\Phi$ C31 to transform E.  
15 coli BL21 $\Delta$ recA.

The spore suspension of *Streptomyces cyaneogriseus* subsp. noncyanogenus  $\Delta$ nemA4::vph attB<sub>TG1</sub>::aveA4-aveA3-aveE obtained in example 2 was inoculated into YEME medium (500 ml Erlenmeyer  
20 flask) containing 50 ml of 30% w/v sucrose, 0.5% w/v glycine and 5mM MgCl<sub>2</sub>, and cultured at 30°C for 48 hours in the rotary shaker. Mycelia were collected by centrifugation at 3000 rpm for 10 min. After P10 medium 20 ml was added and suspended well, the suspension was centrifuged at 3000 rpm for 10 min. to wash  
25 mycelia. The P10 medium containing egg lysozyme 1 mg/ml was added to the washed mycelia to suspend and to generate the protoplast by keeping at 30°C for 30 minutes. After well mixing with adding P10 medium 10 ml, the protoplast suspension was passed through a cotton plug filter to remove lysozyme indigested mycelia. The

protoplast suspension which was passed through the cotton plug filter was centrifuged at 3000 rpm for 10 min. to precipitate the protoplast. After removing the supernatant and suspending well with P10 medium 10 ml, the protoplast was precipitated by centrifuging at 3000 rpm for 10 min. The P10 medium 10 ml was again added thereto and the protoplast was suspended and washed by centrifugation. The obtained washed protoplast was suspended in P10 medium 5 ml. The suspension, each 0.1 ml, was dispensed in the sterilized Eppendorf tube and stored at -80°C. The plasmid DNA pUC19aad3"-int $\Phi$ C31::aveR, about 1  $\mu$ g, obtained hereinabove was added to the protoplast hereinabove and immediately added and mixed with 25% polyethylene glycol MW1000 solution (2.5% sucrose, 0.05% KH<sub>2</sub>PO<sub>4</sub>, 0.1 M CaCl<sub>2</sub> and 50mM Tris-maleate, pH 8.0) 500  $\mu$ l and allowed to stand at room temperature for 1 min.

15

After P10 medium 450  $\mu$ l was added and mixed well, each 100  $\mu$ l thereof was placed on R2YE agar medium and was spread over together with soft agar medium 2.5 ml. After incubating at 30°C for 20 hours, a soft agar medium 2.5 ml containing spectinomycin 3 mg/ml was overlaid. The medium was cultured at 30°C for 5 days to obtain the transformant resistant to spectinomycin. The transformant resistant to spectinomycin grown on the surface of R2YE agar medium was spread aseptically over the YMS agar medium containing spectinomycin 300  $\mu$ g/ml. The thus obtained each strain was referred to *Streptomyces cyaneogriseus* subsp. noncyanogenus  $\Delta$ anmA4::vph attB<sub>TG1</sub>::aveA4-aveA3-aveE attB $\phi$ C31::aveR.

25

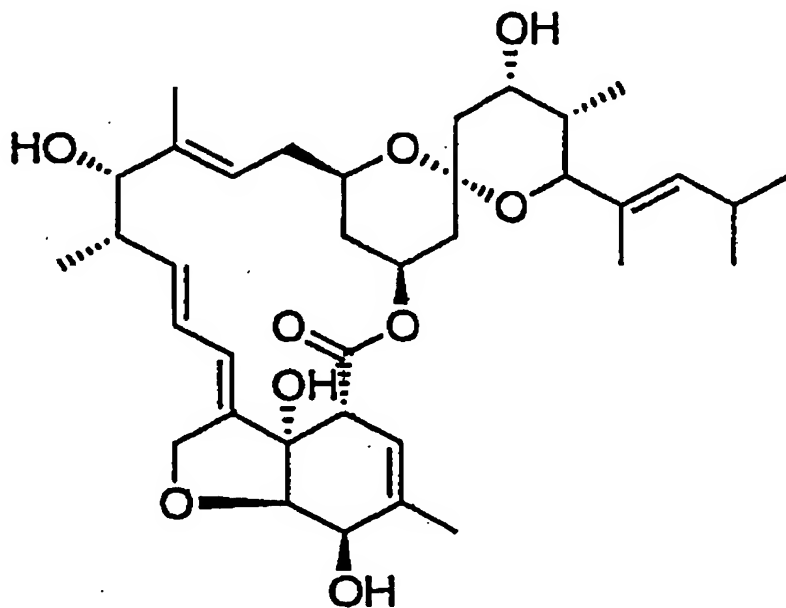
#### Example 4

Culturing *Streptomyces cyaneogriseus* subsp. *noncyanogenus*  
 $\Delta$ nemA4::vph attB<sub>TG1</sub>::aveA4-aveA3-aveE attB<sub>φC31</sub>::aveR and  
isolation and purification of the product

The strain integrated with aveA3-4 and aveR in the strain  
5 inserted with vph of nemadectin PKS module 7 on the chromosome  
obtained in example 3 was inoculated in a nemadectin seed culture  
medium and cultured at 30°C for 3 days. The cultured medium 1  
ml was added to the nemadectin production medium 50 ml dispensed  
in the 500 ml Erlenmeyer flask. This was shake cultured at 28°C  
10 for 5 days under 180 rpm and centrifuged at 3000 rpm for 10 min.  
to obtain mycelia. The obtained mycelia were suspended with  
acetone, stirred at room temperature for 1 hour and the mycelia  
and acetone layer were collected separately. The solvent was  
distilled from the acetone layer. Water and chloroform were added  
15 to the substance dried by distillation of the solvent and the  
mixture was stirred. Chloroform layer was collected separately  
and sodium sulfate anhydride was added for dehydration. Solvent  
was distilled off from the chloroform layer. The resultant crude  
extract was dissolved in small amount of chloroform. The solution  
20 was charged on a column of silica gel (Sigma-Aldrich Co., U.S.)  
equilibrated with chloroform. After washing the column with  
chloroform, the column was washed with 25% v/v ethyl  
acetate/chloroform to remove fractions without containing C-13  
hydroxyl nemadectin. Subsequently, fractions eluted with 40%  
25 v/v ethyl acetate/chloroform were removed and fractions  
containing large amount of C-13 hydroxyl nemadectin eluted with  
50% v/v ethyl acetate/chloroform were collected. The obtained  
eluate was dried in vacuo to obtain yellowish oily substance.  
The thus obtained yellowish oily substance was isolated and

purified by the following condition using HPLC.

When Pegasil ODS column (ODS: 3  $\mu$ m; column size: 20  $\phi$  mm  
× 250 mm; Senshu Scientific Co., Ltd., Japan) was used under  
5 the condition of the mobile phase of a mixed solvent consisting  
of acetonitrile 50%, methanol 18% and water 32%, detection at  
246 nm and separation at flow rate 8 ml/min., a component with  
the retention time 28 minutes was isolated. The obtained compound  
was analyzed the structure by  $^1\text{H}$ -NMR spectrum (refer to Fig. 3)  
10 data,  $^{13}\text{C}$ -NMR spectrum (refer to Fig. 4) data and mass spectrum  
data ( $M+1 = 629$ ), and was confirmed to be C-13 hydroxyl nemadectin  
 $\alpha$  (molecular formula:  $\text{C}_{36}\text{H}_{52}\text{O}_9$ ) represented by the following  
formula.



15

#### Example 5

Obtaining avermectin glycosidation genes aveBI-BVIII derived  
from *Streptomyces avermitilis*

A DNA fragment, 11041 bp, described in SEQ ID NO:6, i.e.

pUC19::aveBI-BVIII ligated with DNA containing total aveBI-BVIII, was digested with restriction enzymes XbaI and HindIII, and the DNA fragment containing total aveBI-BVIII was electrophoresed with low melting point agarose gel.

5

The gel was isolated and purified by means of phenol extraction, phenol-chloroform extraction and alcohol precipitation. In addition, a chromosome inserted vector plasmid pUC19intR4-tsr was digested with the restriction enzyme XbaI-HindIII and electrophoresed with agarose gel to isolate and purify DNA fragment, 11 kb. DNA fragments were ligated by using Ligation High, and E. coli BL21ΔrecA was transformed with using the DNA ligation product 10 μl to obtain the recombinant plasmid pUC19intR4-tsr::aveBI-BVIII. A selection of the transformant was performed by using LB medium containing ampicillin 50 μg/ml.

#### Example 6

Introduction of avermectin glycosidation and oleandrose biosynthesis genes aveBI-BVIII derived from Streptomyces avermitilis into Streptomyces cyaneogriseus subsp. noncyanogenus ΔnemA4::vph attB<sub>TG1</sub>::aveA4-aveA3-aveE attB<sub>φC31</sub>::aveR

Spore suspension of Streptomyces cyaneogriseus subsp. noncyanogenus ΔnemA4::vph attB<sub>TG1</sub>::aveA4-aveA3-aveE attB<sub>φC31</sub>::aveR obtained in example 3 was inoculated into YEME medium (500 ml Erlenmeyer flask) containing 50 ml of 30% w/v sucrose, 0.5% w/v glycine and 5mM MgCl<sub>2</sub>, and cultured at 30°C for 48 hours in the rotary shaker. Mycelia were collected by centrifugation at 3000 rpm for 10 min. After P10 medium 20 ml



was added and suspended well, the suspension was centrifuged at 3000 rpm for 10 min. to wash mycelia. The P10 medium containing egg lysozyme 1 mg/ml was added to the washed mycelia to suspend and to generate the protoplast by keeping at 30°C for 30 minutes.

5 After well mixing with adding P10 medium 10 ml, the protoplast suspension was passed through a cotton plug filter to remove lysozyme indigested mycelia. The protoplast suspension which was passed through the cotton plug filter was centrifuged at 3000 rpm for 10 min. to precipitate the protoplast. After

10 removing the supernatant and suspending well with P10 medium 10 ml, the protoplast was precipitated by centrifuging at 3000 rpm for 10 min. The P10 medium 10 ml was again added thereto and the protoplast was suspended and washed by centrifugation. The obtained washed protoplast was suspended in P10 medium 5

15 ml. The suspension, each 0.1 ml, was dispensed in the sterilized Eppendorf tube and stored at -80°C. The plasmid DNA pUC19intrR4-tsr::aveBI-BVIII, about 1 µg, obtained in example 5 was added to the protoplast hereinabove and immediately added and mixed with 25% polyethylene glycol MW1000 solution (2.5%

20 sucrose, 0.05% KH<sub>2</sub>PO<sub>4</sub>, 0.1 M CaCl<sub>2</sub> and 50mM Tris-maleate, pH 8.0) 500 µl and allowed to stand at room temperature for 1 min.

After P10 medium 450 µl was added and mixed well, each 100 µl thereof was placed on R2YE agar medium and was spread over

25 together with soft agar medium 2.5 ml. After incubating at 30°C for 20 hours, a soft agar medium 2.5 ml containing thiostrepton 200 µg/ml was overlaid. The medium was cultured at 30°C for 5 days to obtain the transformant resistant to thiostrepton. The transformant resistant to thiostrepton grown on the surface of

R2YE agar medium was spread aseptically over the YMS agar medium containing thiostrepton 20 µg/ml. The thus obtained each strain was referred to *Streptomyces cyaneogriseus* subsp. *noncyanogenus*  $\Delta$ nemA4::vph attB<sub>TG1</sub>::aveA4-aveA3-aveE attB<sub>ϕC31</sub>::aveR  
5 attB<sub>R4</sub>::aveBI-BVIII.

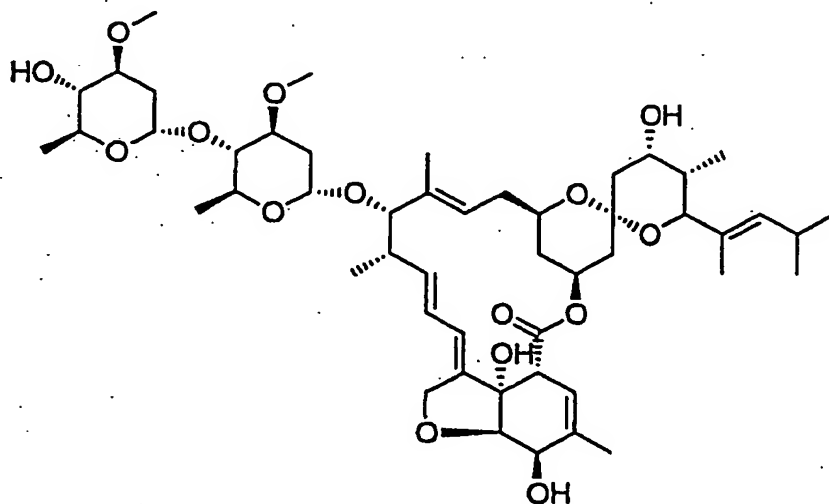
#### Example 7

Culturing *Streptomyces cyaneogriseus* subsp. *noncyanogenus*  $\Delta$ nemA4::vph attB<sub>TG1</sub>::aveA4-aveA3-aveE attB<sub>ϕC31</sub>::aveR  
10 attB<sub>R4</sub>::aveBI-BVIII and isolation and purification of product

The strain integrated with aveA3-4, aveR and aveBI-BVIII in the strain inserted with vph of nemadectin PKS module 10 on the chromosome obtained in example 6 was inoculated in a nemadectin seed culture medium and cultured at 30°C for 3 days.  
15 The cultured medium 1 ml was added to the nemadectin production medium 50 ml dispensed in the 500 ml Erlenmeyer flask. This was shake cultured at 28°C for 5 days under 180 rpm and centrifuged at 3000 rpm for 10 min. to obtain mycelia. The obtained mycelia were suspended with acetone, stirred at room temperature for  
20 1 hour and the mycelia and acetone layer were collected separately. The solvent was distilled from the acetone layer. Water and chloroform were added to the substance dried by distillation of the solvent and the mixture was stirred. Chloroform layer was collected separately and sodium sulfate  
25 anhydride was added for dehydration. Solvent was distilled off from the chloroform layer. The resultant crude extract was dissolved in small amount of chloroform. The solution was charged on a column of silica gel (Sigma-Aldrich Co., U.S.) equilibrated with chloroform. After washing the column with chloroform, the

column was washed with 30% v/v ethyl acetate/chloroform to remove fractions without containing C-13 glycosylnemadectin. Subsequently, fractions eluted with 40% v/v ethyl acetate/chloroform and 50% v/v ethyl acetate/chloroform were collected. Each obtained eluate was dried in vacuo to obtain yellowish oily substance. The thus obtained yellowish oily substance was isolated and purified by the following condition using HPLC.

Pegasil ODS column (3  $\mu$ m; column size: 20  $\phi$  mm  $\times$  250 mm; Senshu Scientific Co., Ltd., Japan) was used and a solvent mixture in a ratio of acetonitrile : methanol : water = 55 : 18 : 27 was used as the mobile phase. A flow rate was set at 6 ml/min., and a component having the retention time 120 minutes was collected with the indication of absorption at 246 nm. The obtained compound was analyzed the structure by  $^1\text{H}$ -NMR spectrum (refer to Fig. 5) data,  $^{13}\text{C}$ -NMR spectrum (refer to Fig. 6) data and mass spectrum data ( $M+1 = 917$ ), and was confirmed to be C-13 glycosylnemadectin  $\alpha$  (molecular formula:  $\text{C}_{50}\text{H}_{76}\text{O}_{15}$ ) represented by the following formula.



Compositions of various media and buffer used in examples hereinabove are shown as follows.

5 Buffer for dilution of phage (SM buffer)

Tris-HCl (ph 7.5)	10 mM
Sodium chloride	100 mM
Magnesium sulfat $e$ 7H $_2$ O	10 mM

10 Solution for terminating cycle sequencing reaction

Bromophenol Blue	0.02%
EDTA (pH 8.0)	20 mM
Formamide	95%

15 YEME medium

Yeast extract (Difco Laboratories)	3 g
Malt extract (Oxoid Ltd.)	3 g
Peptone (Difco Laboratories)	5 g
Glucose	10 g

20 Sucrose	300 g
Distilled water	1000 ml

pH not adjusted, 121°C, high pressure steam sterilization for 1 min.

25 Trace element solution

Ferric chloride 6 hydrate	200 mg
Zinc chloride	40 mg
Cupric chloride 2 hydrate	10 mg
Manganese chloride 4 hydrate	10 mg

Sodium borate 10 hydrate	10 mg
Ammonium molybdate 4 hydrate	10 mg
Distilled water	1000 ml

5 P10 medium

Sucrose	103 g
Potassium sulfate	0.25 g
Magnesium chloride 6 hydrate	2.03 g
Trace elements solution	2.0 ml

10 After high pressure steam sterilization at 121°C for 15 min., following components are aseptically added.

0.5% potassium phosphate	10 ml
3.68% potassium chloride 2 hydrate	100 ml
0.25 M TES* (pH 7.2)	100 ml

15 \* N-tris(hydroxymethyl)methyl-2-aminoethanesulfonate

R2YE agar medium

Sucrose	103 g
Potassium sulfate	0.25 g
Magnesium chloride 6 hydrate	10.12 g
Glucose	10 g
Casamino acid (Difco Laboratories)	0.1 g
Agar	22 g
Distilled water	800 ml

25 After high pressure steam sterilization at 121°C for 15 min., following components are aseptically added.

Trace elements solution	2 ml
0.5% potassium phosphate	10 ml
3.68% potassium chloride 2 hydrate	80 ml

20% L-proline	15 ml
0.25M TES (pH 7.2)	100 ml
10% yeast extract (Difco Laboratories)	50 ml
1M sodium hydroxide	5 ml

5

#### Soft agar medium

Sucrose	103 g
Magnesium chloride 6 hydrate	10.12 g
Agar (Difco Laboratories)	6.5 g

10	Distilled water	820 ml
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After high pressure steam sterilization at 121°C for 15 min., following components are aseptically added.

3.68% potassium chloride 2 hydrate	80 ml
0.25 M TES (pH 7.2)	100 ml

15

#### YMS agar medium

Malt extract (Difco Laboratories)	10 g
Yeast extract (Difco Laboratories)	4 g
Soluble starch (Difco Laboratories)	4 g

20	Agar	20 g
	Distilled water	1000 ml

After adjusting pH 7.4 by adding 2M potassium hydroxide, subjecting to high pressure steam sterilization at 121°C for 15 min. After the sterilization, magnesium chloride and 25 potassium nitrate were added to be 10 mM and 8 mM, respectively.

#### LA medium

Tryptone (Oxoid Ltd.)	10 g
Yeast extract (Oxoid Ltd.)	5 g

Sodium chloride	5 g
Agar	15 g
Distilled water	1000 ml

After adjusting pH 7.2 by adding 2M potassium hydroxide,  
 5     subjecting to high pressure steam sterilization at 121°C for  
 15 min.

#### Seed culture medium for nemadectin producing strain

	Glucose	10 g
10	Dextrin	20 g
	Yeast extract	5 g
	NZ-amine A	5 g
	Calcium carbonate	1 g
	Distilled water	1000 ml

15         pH not adjusted. High pressure steam sterilization at 121°C  
 for 15 min.

#### Culture medium for nemadectin production

	Glucose	50 g
20	Cotton seed powder	25 g
	Calcium carbonate	7 g
	Distilled water	1000 ml

pH not adjusted. High pressure steam sterilization at 121°C  
 for 15 min.

25

#### Industrial Applicability

As described hereinabove, the present invention relates  
 to the invention comprising introducing DNA of the nemadectin  
 analogous compound producing microorganism into the nemadectin

producing microorganism belonging to genus Streptomyces, producing and accumulating C-13 hydroxyl nemadectin and C-13 glycosylnemadectin, and collecting the same. The stereoselectively glycosidated nemadectin derivatives can be  
5 effectively obtained by preparing C-13 glycosylnemadectin by means of molecular genetic technology. Improvements in biological activities such as anti-insects and anti-parasites can be expected.